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Oxytocin-induced cervical dilation and cervical manipulation in sheep: Effects on laparoscopic artificial insemination[1]

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ABSTRACT: The difficulty of cervical penetration severely limits the use of transcervical AI (TAI) in sheep, and trauma from cervical manipulation (CM) may reduce fertility after TAI. We investigated the effects of cervical dilation using exogenous oxytocin (OT) to facilitate TAI and its effects on reproductive variables after laparoscopic AI (LAI). Estrus was synchronized by inserting pessaries impregnated with 6α-methyl-17α-hydroxyprogesterone acetate (60 mg) for 12 d. In Exp. 1, we determined whether OT and CM before LAI affected the interval from pessary removal to ovulation and fertilization rate. Crossbred ewes (n = 16) were assigned to 1) saline-CM or 2) OT-CM. In Exp. 2, effects of OT and CM on lambing rates were evaluated with white-faced ewes (n = 220) in a 2 × 2 factorial experiment: 1) saline-sham CM; 2) saline-CM; 3) OT-sham CM; and 4) OT-CM. In both studies, eCG (400 IU i.m.) was injected at pessary removal, and LAI was performed 48 to 52 h later. In Exp. 1, ewes received i.v. either 400 USP units of OT or 20 mL of saline at 30 to 60 min before LAI, and CM was administered as for TAI. Beginning 32 h after pessary removal and continuing at 8-h intervals, ovaries were examined with ultrasonography to estimate time of ovulation. Treatment in Exp. 1 did not affect combined ovum/embryo recovery rate (69%), but OT-CM decreased fertilization rate (47 vs 59%; $P < 0.05$). The OT tended to reduce the interval to ovulation (OT, 59 h vs saline, 66 h; $P < 0.06$). The OT × CM interaction in Exp. 1 was not significant. For Exp. 2, approximately 25 min before sham CM or CM, 200 USP units of OT or 10 mL of saline was injected i.v. The LAI was performed immediately after sham CM or CM. At 10 to 12 d after AI in Exp. 2, ewes were mated with Suffolk rams. Blood was collected between 24 and 26 d after AI for pregnancy-specific protein B (PSPB) RIA. The PSPB pregnancy and lambing rates were both 62% in saline-sham controls. The CM did not affect pregnancy (69%) or lambing rate (64%). The OT treatment decreased ($P < 0.05$) PSPB pregnancy (59%) and lambing rates (56%) in OT-sham ewes and pregnancy and lambing rates in CM ewes (both 43%). Neither CM nor OT before LAI affected lambing rates to next estrus, indicating no long-term damage to the cervix or uterus. In summary, CM did not affect fertility after LAI, but OT decreased lambing rate independent of CM. If OT will not be usable for TAI, it may still be a tool for training TAI personnel.

Key Words: Artificial Insemination, Cervix, Laparoscopy, Oxytocin, Sheep

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Introduction

The unusual anatomy of the cervix in ewes prevents routine passage of transcervical (**TC**) instruments (Fukui and Roberts, 1976; Bunch and Ellsworth, 1981). The size and shape of the external cervical os and the cervical canal present the major barriers to TC-AI (Dun, 1955; Andersen et al., 1973; Halbert et al., 1990), preventing widespread use of AI in sheep. A feasible TC-AI method for sheep must include a method for coping with anatomical challenges of the cervix without inducing trauma. Our previous work indicated that oxytocin (**OT**) treatment induced cervical dilation and decreased the difficulty of passing a catheter through the cervix and into the uterus (Khalifa et al., 1992; Sayre and Lewis, 1997). Despite that, there are several unknown factors associated with this treatment. Even though OT did not affect fertilization rate, effects of cervical manipulation or effects of the overall treatment (i.e.,

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OT-cervical manipulation) have not been assessed (Sayre and Lewis, 1997). Decreases in lambing rates have been associated with cervical manipulation. This study was conducted to determine whether an OT treatment could be used to assist TC passage of an AI instrument and to determine whether this affected the outcome of laparosopic AI during the fall breeding season.

Materials and Methods

Estrus Synchronization and Artificial Insemination

Similar estrus synchronization protocols were used in Exp. 1 at Virginia Tech (Blacksburg) and in Exp. 2 at the USDA-ARS U.S. Sheep Experiment Station (**USSES**; Dubois, ID) during the fall breeding period. Pessaries containing 60 mg of 6α-methyl-17α-hydroxyprogesterone acetate (Tuco Products Limited, Orangeville, Ontario, Canada) were inserted. Six days after pessaries were inserted, ewes in Exp. 1 received a 5-mg i.m. injection of Lutalyse (Pharmacia & Upjohn, Kalamazoo, MI) and a second 5-mg injection 4 to 6 h later to eliminate corpora lutea. Pessaries were removed after 11 (Exp. 1) or 12 d (Exp. 2). At pessary removal, 400 IU of eCG (Sioux Biochemical, Sioux City, IA) were injected i.m. For Exp. 1, ovarian ultrasonography was performed to determine time from pessary removal to ovulation, so estrus was not monitored. In Exp. 2, ewes were checked for estrus at 24, 36, and 48 h after pessary removal, using rams fitted with aprons to prevent breeding. Ewes standing firmly to be mounted were considered to be in estrus. If ewes were not observed in estrus, their data were removed from the main data set. For Exp. 1 and 2, semen for AI was collected with an artificial vagina from white-faced rams of known fertility at the USSES. Semen from several rams with individual post-thaw motilities greater than 40% was combined to reduce ram variation within and between the two experiments. Semen was frozen (Rodriguez, 1991; Rodriguez et al., 1993) and stored until it was used for AI. At the time of AI, semen was thawed, and post-thaw motility and forward progressive movement were assessed. The frozen-thawed semen contained from 50×10^6 to 75×10^6 spermatozoa per 200 μL and averaged greater than 40% progressive motility.

Ewes were inseminated with a standard laparoscopic AI procedure (Evans and Maxwell, 1987; Stellflug et al., 1991) 48 to 52 h after pessary removal. All AI was laparoscopic so that the effects of treatments would be independent of the AI procedure.

Cervical Manipulation

Ewes were restrained in a Poldenvale Commodore chute (Premier Sheep Supplies, Washington, IA) in a dorsal recumbent position. Wool was shorn around the perineal area 2 h (Exp. 1) and 48 to 72 h (Exp. 2) before treatment. The perineal area was scrubbed with an antiseptic soap and rinsed thoroughly. Excess water and antiseptic were removed with dry gauze sponges. A coating of obstetrics lubricant was applied to a tubular speculum, and the speculum was inserted into the vagina and pushed against tissue surrounding the cervix. If the external cervical os was not in the center of the speculum lumen, a cattle AI catheter was placed into the folds of tissue surrounding the external cervical os to position the cervix. After the cervix was centered, the cattle AI catheter was removed, and the TC-AI catheter was placed at the external cervical os and manually manipulated through the cervix. A TC-AI catheter used for cervical manipulation in this experiment was identical to the TC embryo transfer instrument described previously (Wulster-Radcliffe et al., 1999). The tip of the catheter was believed to have passed through the cervix when cervical rings no longer obstructed it or when there was a change in tissue tone between the cervix and uterus. Ultrasonography has been used to confirm the validity of this assessment (Wulster-Radcliffe et al., 1999). The time required for cervical passage was recorded. To mimic a typical TC-AI protocol, the TC-AI catheter was left in the uterus for 2 min following passage through the cervix.

Experiment 1

The aim of Exp. 1 was to evaluate the effects of an exogenous OT treatment and cervical manipulation on the interval from pessary removal to ovulation, fertilization rate, and early embryo quality. Crossbred multiparous ewes from the Virginia Tech Sheep Center were randomly assigned to one of two treatment groups: 1) saline-cervical manipulation and 2) OT-cervical manipulation. Eight ewes were assigned to each treatment group. Thirty to 60 min before laparoscopic AI, ewes received i.v. either 400 USP units of OT (Phoenix Scientific, St. Joseph, MO) or 20 mL of saline (Khalifa et al., 1992). The cervix was manipulated (i.e., TC passage of an AI catheter approximately 20 min after OT injection). Immediately after cervical manipulation, ewes were anesthetized with 40 mg of xylazine (Rompun, Bayer Corp., Shawnee Mission, KS) i.m. and sodium pentobarbital in saline (< 5 mL of 65 mg/mL i.v.; Sigma Chemical Co., St. Louis, MO) and artificially inseminated laporoscopically.

Transcrectal Ultrasonography. Ovaries were evaluated using a transrectal ultrasonographic procedure (Schrick et al., 1993). An Aloka 500V instrument with a 5.0-MHz linear-array transducer was used (Corometrics Medical Systems, Wallingford, CT). Polyvinyl chloride tubing (1.4 cm i.d., 2 cm o.d., and 30 cm long) was used to sheathe the cable, to hold the transducer in a fixed position, and to provide a means for manipulating the transducer in the rectum. Ovaries were evaluated beginning 32 h after pessary removal and then at 8-h intervals until there was evidence of ovulation, that is, the appearance of the largest follicle(s) changed from a uniform dark gray, which is associated with a fluid-

filled structure, to mottled shades of gray with a faint outline of the original structure. The caliper function in the instrument was used to measure the sizes (i.e., greatest distance across a follicle) of the largest follicles at each evaluation, and ovulation rate was determined. The same chute that was used for transcervical manipulation was used to restrain the ewes for this procedure. Because ultrasound evaluations around the time of ovulation were at 8-h intervals, the interval from pessary removal to ovulation was estimated by subtracting 4 h from the time that ovulation was detected.

Embryo/Ovum Collection. Embryos and(or) ova were collected 72 h after AI. The uterus was exposed through a midventral laparotomy (Hunter et al., 1955), a catheter was inserted into an oviduct, and 12 mL of sterile PBS was injected into the ipsilateral uterine horn anterior to the uterine body. The PBS was massaged toward the tip of the uterine horn, into the oviduct, through the catheter, and into a Petri dish to recover embryos and(or) ova. Corpora lutea (**CL**) were counted at the time of embryo/ovum recovery. Embryos were held in PBS while the number of embryos and unfertilized ovum were counted. Recovery rate ([embryos + ova] ÷ number of CL) was determined. Embryos were assessed morphologically and were graded 1 through 4 based on the organization and consistency in shape and size of the blastomeres. Embryos that were degenerate were Grade 1, and embryos that appeared to have undergone successive mitotic divisions (i.e., somewhat uniform blastomeres) were Grade 4 and considered excellent. Grades 2 and 3 were considered below average and very good, respectively. Unfertilized ova were not assigned a score. Embryos were further divided categorically. Four-celled embryos were considered Stage 1 of development, and eight-celled embryos were considered Stage 2.

Experiment 2

The aim of Exp. 2 was to evaluate effects of an exogenous OT treatment and cervical manipulation on lambing rates. White-faced multiparous ewes (n = 220) from USSES flocks were assigned to one of four randomized treatments in a 2 × 2 factorially designed experiment: 1) saline-sham cervical manipulation; 2) saline-cervical manipulation; 3) OT-sham cervical manipulation; and 4) OT-cervical manipulation. Oxytocin (200 USP units) or an equivalent volume of saline (10 mL) was administered i.v. approximately 25 min before cervical manipulation (i.e., TC passage of the AI catheter) or a sham cervical manipulation. This lower dose of OT, compared with that in Exp. 1, was selected because of the difficulty of injecting 20 mL i.v. Based on a dose titration experiment (Sayre and Lewis, 1996), 200 USP units of OT was as effective as 400 USP units for dilating the cervix. Ewes receiving sham treatment were restrained in a dorsal recumbent position in a Poldenvale Commodore chute. A speculum was inserted into the vagina, and the cervix was observed. Ewes were artificially inseminated laparoscopically immediately after cervical manipulation or sham manipulation. For laparoscopic AI, ewes were anesthetized with 100 to 150 mg of ketamine (Vedco, St. Joseph, MO). After AI, each ewe received 10 mL of penicillin (300,000 IU/mL; Vedco) and 1 g of phenylbutazone (Vedco). Ten to 12 d after AI, ewes were placed with fertile Suffolk rams for 21 d. The Suffolk rams, and thus mottled-faced lambs, aided in determining whether a ewe was pregnant from AI.

Pregnancy-Specific Protein B RIA. Serum was assayed for pregnancy-specific protein B (**PSPB**; Ruder et al., 1988; Sasser et al., 1986; Willard et al., 1995). Intra- and interassay CV were 7.7 and 20.3%, respectively. Ewes that were positive for PSPB were considered pregnant. Blood samples for PSPB were taken 24 to 26 d after AI to ensure that a positive determination of PSPB could be attributed to a pregnancy from the AI rather than from matings with the Suffolk rams. Initial pregnancy rates were determined using the PSPB data (ewes within a group positive for PSPB ÷ total number of ewe within a group).

Lambing. Ewes were lambed at the USSES under 24-h surveillance. Shortly after lambing, each ewe and her lambs were identified. Face color of the lambs and date of lambing were used to determine breed of sire.

Statistical Analyses. In Exp. 1, the GLM procedures of SAS (SAS Inst. Inc., Cary, NC) were used to determine the effect of treatment (saline vs OT) on combined ovum and embryo recovery rate and on the interval from pessary removal to ovulation. The GENMOD procedures of SAS were used to determine the effect of treatment on fertilization rates. Numbers of embryos, ova, and CL, and embryo/ovum recovery and fertilization rates (embryos ÷ [embryos + ova]) were evaluated on a per-ewe basis. Stage of embryo development and embryo grade were evaluated on a per-ovum basis. In Exp. 2, GENMOD procedures of SAS were used to determine whether OT, cervical manipulation, or the OT × cervical manipulation interaction were significant for PSPB pregnancy rate or lambing rate (ewes lambing from AI ÷ total number of ewes). The GENMOD procedure was also used to determine whether the time of estrus (24, 36, or 48 h after pessary removal) affected lambing rate.

Results

Experiment 1

Fertilization rates ($P < 0.05$) after the OT-cervical manipulation treatment were less ($P < 0.05$) than after saline-cervical manipulation (47 vs 59%). However, OT treatment did not affect the time of ovulation, the number of CL counted at the 72-h embryo/ovum collection time after LAI, or the numbers of ova and embryos recovered. Treatment with oxytocin or saline before cervical manipulation and LAI did not affect embryo grade or stage of development (Table 1).

Table 1. Reproductive responses of ewes treated with oxytocin before cervical manipulation and laparoscopic AI in Exp. 1[a]

	Treatment		
Variable[b]	Saline (n = 8)	Oxytocin (n = 8)	SEM
Recovery, %[c]	89.5 (17/19)	60 (15/25)	0.1
Fertilization, %[de]	58.8 (10/17)	46.6 (7/15)	
No. of ova	2.1	1.9	0.2
No. embryos	1.3	0.9	0.2
Embryo grade[f]	3.4	3.3	0.5
Stage[g]	1.5	1.3	0.1
No. of CL	2.4	3.1	0.3
Time to ovulation, h[h]	66	59	1.8

[a]Ewes were assigned to one of two treatment groups: saline-cervical manipulation or oxytocin (400 USP i.v.)-cervical manipulation. Corpora lutea (CL) were counted at the time embryos and(or) ova were collected 72 h after laparoscopic AI.
[b]Except for embryo grade, each variable was evaluated on a per-ewe basis. Embryo grade was evaluated on a per-embryo basis.
[c]Recovery rates [(embryos + ova) ÷ number of CL] × 100.
[d]Fertilization rates [embryos ÷ (embryos + ova)] × 100.
[e]Data were analyzed with GENMOD procedures. Main effect of oxytocin was significant ($P < 0.05$).
[f]Grade 4 = excellent and Grade 1 = degenerate.
[g]Stage 1 = four-celled embryo; Stage 2 = eight-celled embryo.
[h]Time to ovulation, ± 4 h. Oxytocin tended ($P < 0.06$) to reduce the interval to ovulation.

Experiment 2

Cervical manipulation did not affect PSPB pregnancy rate or lambing rate (Table 2; $P = 0.38$). However, OT treatment decreased ($P < 0.05$) the initial pregnancy rate (i.e., PSPB determination) and the lambing rate (Table 2). The OT × cervical manipulation interaction was not significant (Table 2; $P = 0.38$). Time of estrus had no effect on lambing rate after laparoscopic AI at 48 to 52 h after pessary removal (Table 2; $P = 0.32$). Of the ewes that were not detected in estrus (n = 26), 23% lambed after laparoscopic AI. In ewes that were not pregnant following AI (n = 96), neither cervical manipulation nor OT treatment affected the pregnancy rate (range of 85 to 91%) from natural matings during the subsequent estrous cycle.

Discussion

Previous studies indicate that transcervical intrauterine AI can be extremely difficult in ewes, often resulting in decreased fertilization and pregnancy rates (Eppleston and Maxwell, 1993). Although this has not been quantified, these decreases have been attributed to increased cervical and uterine trauma as a result of cervical and uterine manipulation. The experiments described in this article indicate that atraumatic cervical manipulation does not affect time of ovulation, fertilization rate, early embryonic development, or lambing rate. However, the OT treatment used to dilate the cervix and increase the ease of passage of a transcervical AI instrument through the cervix and into the uterus decreased fertilization rate, pregnancy rate, and lambing rate.

Dilation of the cervix with pharmacological doses of OT at estrus dramatically reduces the difficulty of traversing the cervix (Khalifa et al., 1992). However, the OT treatment used to dilate the cervix and increase the ease of traversing the cervix decreased fertilization rate in Exp. 1 and PSPB pregnancy and lambing rates in

Table 2. Reproductive responses of ewes treated with or without oxytocin before cervical manipulation or sham manipulation and laproscopic AI (LAI) in Exp. 2[ab]

	Cervical manipulation		Oxytocin USP units	
Item	Without	With	0	200
PSPB pregnancy rate, %[cd]	60.5	56.0	65.5	51.0
Lambing rate, %[ef]	59.0	53.5	63.0	49.5
Lambing rate by time of estrus[g]				
24 h	69.0	62.5	67.5	64.0
36 h	61.5	59.0	64.0	56.5
48 h	51.5	48.5	73.5	26.5
Lambing rate to breeing by Suffolk rams[h]	88.0	90.5	91.0	87.5

[a]Ewes in synchronized estrus were assigned to one of four treatment groups: saline-sham cervical manipulation; saline-cervical manipulation; oxytocin-sham cervical manipulation; or oxytocin-cervical manipulation. Oxytocin dose was 200 USP units given i.v. approximately 25 min before cervical manipulation or sham cervical manipulation followed immediately by LAI at 48 to 52 h after pessary removal.
[b]Data were analyzed with GENMOD procedures.
[c]PSPB (pregnancy-specific protein B) pregnancy rate = (ewes positive for PSPB ÷ total number of ewes) × 100.
[d]Main effect of oxytocin was significant ($P < 0.05$).
[e]Lambing rate = (ewes lambing from AI ÷ total number of ewes) × 100.
[f]Main effect of oxytocin was significant ($P < 0.05$).
[g]Influence of the time of estrus (24, 36, or 48 h) after pessary removal on lambing rate after timed LAI. Time of estrus did not affect lambing rate.
[h]Lambing rate ([ewes lambing from matings to Suffolk rams ÷ total number of nonpregnant ewes] × 100) after exposure to Suffolk rams if ewes did not become pregnant after LAI.

Exp. 2. As a result of the effect of OT, the lambing rate was decreased in ewes receiving both treatments (OT and CM) in Exp. 2.

Because of the short half-life of OT, we did not believe that it would have long-term detrimental effects on pregnancy and lambing rates. In previous experiments, the OT treatment used to dilate the cervix in Exp. 1 and 2 induced myometrial tetany, but it did not affect sperm transport to the oviducts (Sayre and Lewis, 1996). Thus, we concluded that other factors should be evaluated to determine whether they could provide greater insights into the effects of our OT treatment. With this in mind, Exp. 1 was conducted to investigate the effects of OT treatment before cervical manipulation on the time of ovulation, fertilization rate, and early embryonic development. Oxytocin did not affect the time of ovulation. However, it did reduce fertilization rate, evaluated at 72 h after AI, but the OT did not affect early embryonic development. The reduction in fertility at 72 h after laparoscopic AI does not fully explain the decreases in pregnancy and lambing rates in Exp. 2. Unfortunately, Exp. 2 was not designed to define the timing of the negative influence of OT on pregnancy and lambing rates. Because of the short half-life of OT and the experimental design, it is not clear when the negative influence of OT occurred.

Even though cervical manipulation reduced pregnancy and lambing rates in previous studies (Sayre and Lewis, 1997), there were no deleterious effects of cervical manipulation on any of the variables studied in Exp. 1 or 2. Previously, researchers have attributed the negative effects of cervical manipulation to the secretion of an unknown spermicidal or embryocidal compound (Hawk, 1983). Therefore, we postulated that, if cervical manipulation is capable of inducing the secretion of a spermicidal or embryocidal compound, the effect could be sufficiently potent to alter other processes, such as the timing of ovulation, that might affect fertilization rate or embryonic development. Indeed, several experiments have shown that changes in the time of ovulation are linked to decreases in fertilization rate and embryonic development (Walker et al., 1989; Weitze et al., 1990; Waberski et al., 1994). However, in the absence of a negative effect of cervical manipulation in Exp. 1 or 2 on any of these variables, it is possible to conclude that atraumatic cervical manipulation does not affect events leading to pregnancy. Perhaps the difference in results between this and previous studies is related to decreased cervical trauma associated with more experienced AI technicians and modifications to the TC-AI instruments. Others have reported similar findings associated with more experience (Buckrell et al., 1992; Windsor et al., 1993).

The time that ewes were detected in estrus (24, 36, or 48 h after pessary removal) did not affect lambing rate after the timed (48 to 54 h after pessary removal) laparoscopic AI. This indicated that there would be little, if any, advantage of inseminating laparoscopically according to the time of estrus.

Even though Exp. 2 was not designed to determine whether OT or cervical manipulation affected the outcome of subsequent matings, results of the matings of ewes that did not become pregnant after AI seem to indicate that neither OT nor cervical manipulation had prolonged detrimental effects.

In conclusion, our present results indicate that cervical manipulation as required to traverse the cervix and inseminate directly into the uterus does not interfere with pregnancy or lambing rates, but exogenous OT treatment may decrease fertility, pregnancy, and lambing rates. Despite that, OT seems to be of benefit in traversing the cervix in a timely and atraumatic manner, and it seems to aid in the process of transferring our TC-AI procedure to others.

Implications

Cervical manipulation associated with transcervical artificial insemination (AI) in sheep does not seem to affect fertility after laparoscopic AI or lambing rates after the subsequent estrus in ewes that did not conceive to AI. However, exogenous oxytocin used to facilitate transcervical AI may decrease lambing rates. Nevertheless, oxytocin-induced cervical dilation may aid in the training of AI personnel. Because cervical manipulation alone does not seem to reduce lambing rates, transcervical AI in sheep still seems to be a promising procedure.

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